APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1992, p. 2565–2570
0099-2240/92/082565-06$02.00/0

Vol. 58, No. 8

Contribution of Anaerobic Protozoa and Methanogens to Hindgut Metabolic Activities of the American Cockroach, *Periplaneta americana*

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Received 20 March 1992/Accepted 2 June 1992

The ciliate *Nyctotherus ovalis* occurs in high numbers in the hindgut of the American cockroach (*Periplaneta americana*) and harbors methanogenic bacteria as endosymbionts. The contribution of these hindgut microorganisms to metabolic and developmental processes of *P. americana* was studied by comparing cultures of cockroaches in which the composition of the hindgut microbial population was altered in various ways. Rearing the insects protozoan free resulted in increased insect generation time, decreased adult body weight, and absence of methane production. After feeding of protozoan-free adult cockroaches with a hindgut suspension containing *N. ovalis* and methanogens, methane increased to normal values and insect body weight was restored during the development of the second generation of insects. Feeding the protozoan-free cockroaches a hindgut suspension which was made free of *N. ovalis* resulted in an increase in methane production to only about 20% of the normal methane production level. This suggests that the methanogenic endosymbionts of *N. ovalis* are the major source of methane production in the hindgut. Inhibition of methanogens by addition of bromoethanesulfonic acid to the drinking water of a normal cockroach culture resulted in a reduction of methane production to about 2% of the normal level. No effects on insect body weight or the number of *N. ovalis* organisms were observed, but the fermentation pattern in the hindgut was shifted towards a relative increase in propionate levels. Similar results were obtained for in vitro cultures of hindgut microorganisms treated with bromoethanesulfonic acid. The results suggest a major role for hindgut protozoa in cockroach metabolic activities, especially during the insect growth period. The relatively large amounts of methane produced by cockroaches and by other methane-producing xylophagous insects suggest a major contribution by insects to global methane production.

Most cockroach species are versatile, omnivorous insects which feed on virtually any organic material that is available, including relatively refractory, nitrogen-poor feed components, such as cellulosic plant residues. Although numerous species of microorganisms have been demonstrated in different regions of the cockroach digestive tract (2, 9), little is known about the complex metabolic interactions among microorganisms associated with the cockroach gut. Also, information about the metabolic interactions between gut microorganisms and the insect host is fragmentary and contradictory. Several studies suggest that the presence of the large and diverse microbial population in the cockroach hindgut contributes to nutritional and developmental processes of the host (2–5, 7), but there is no definitive evidence of this (9). Bracke et al. (4), for instance, reported that elimination of the anaerobic hindgut microbiota by a high dose of metronidazole had no effects on the metabolic processes in adult *Periplaneta americana*. More information on dominant hindgut microorganisms and their metabolic activities is needed to assess the role of these organisms in overall cockroach metabolism.

The hindgut has been reported to harbor a more dense and diverse microbial population than the foregut or midgut, a significant portion being firmly attached to the hindgut wall (9). The hindgut provides an anaerobic environment that favors the development of anaerobic microorganisms, including methanogens (5). Although methane production originating from the hindgut of cockroaches has been a well-known phenomenon (9), it was only recently demonstrated that methanogenic bacteria live as endosymbionts of *Nyctotherus ovalis*, a ciliate present in high numbers in the hindgut of the American cockroach, *P. americana* (13). The methanogens appeared to be hydrogenotrophic and were closely associated with ciliate organelles thought to be hydrogenosomes. This suggests that the ciliate-methanogen symbiosis is probably based on interspecies transfer of hydrogen. A similar symbiosis between anaerobic protozoa and methanogens has been reported for rumen ciliates (20, 23), sapropelic ciliates (21, 22), and termite hindgut flagellates (16). The low partial hydrogen pressure brought about by the methanogens, which may be associated as epi- or endosymbionts, allows anaerobic protozoa to produce more oxidized and more energy-yielding fermentation products. This may explain the higher cell densities and faster growth rates which were observed for the flagellate *Trichomitopsis termopsidis* from the termite hindgut when it was cultured in the presence of hydrogenotrophic methanogens (18).

The high cell density (5×10^4 to 6×10^4/ml) and relatively large size (100 to 300 μm) of *N. ovalis* in the cockroach hindgut suggests a substantial contribution of these ciliates to the metabolic activities in the hindgut. The present study was aimed at further examining the role of the ciliate-methanogen symbiotic partners in hindgut metabolic activities, such as production of methane and volatile fatty acids (VFA), and also in overall cockroach performance. The

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effects of elimination of and subsequent reinfection with methanogenic bacteria and/or gut protozoa were studied.

MATERIALS AND METHODS

Insects. Cultures of the American cockroach (*P. americana*) were maintained in 5-liter glass containers as previously described (13). Six different cultures of cockroaches were used in this study. Culture I was a stock culture. Culture II was reared free of protozoa. Culture III was a subculture of culture II that was infected with hindgut microbiota from culture I. Culture IV was a subculture of culture II that was infected with a hindgut suspension devoid of protozoa. Culture V was a subculture of culture I whose drinking water contained 25 mM bromoethanesulfonic acid (BES). Culture VI was a subculture of culture I whose drinking water contained 50 mM BES. Each cockroach culture was kept in duplicate. All cultures were fed a mixture consisting of maize flour, fish flour prepared from *Rastineobola argentea*, and ground cellulose tissue (weight ratio, 1:2:6). The maize and fish flour were obtained from local market places in Dar es Salaam, Tanzania. Water and feed were added ad libitum to separate petri dishes which were refreshed twice every week. The containers with cockroaches were kept in the dark at room temperature (25 to 28°C).

Culture conditions. (i) The stock culture (culture I). The stock culture had been maintained in the laboratory for more than 1 year when subcultures II to VI were derived from this stock. The origin of the insects was described previously (13).

(ii) Cockroaches free of *N. ovalis* (culture II). Cockroaches free of *N. ovalis* were obtained from eggs (oothecae) taken from culture I which were thoroughly washed in a mineral buffer solution described previously (14) to remove possible contamination of gut microorganisms from the stock culture. When the eggs hatched, the nymphs were kept on the same feed used for the stock culture. The substrates were autoclaved before addition to the culture, but no other attempts were made to keep the environment aseptic. The absence of *N. ovalis* was routinely checked during the development of the insects and in adults by studying hindgut samples microscopically. After 3.5 months, when the female insects began to produce oothecae, the culture was split into three subcultures (II, III, and IV), each containing 20 adult individuals.

(iii) Infection with a total hindgut microbial population (culture III). To test the effect of reinfection of adult, ciliate-free cockroaches derived from culture II with mixed hindgut microbiota, 20 adults were transferred into a clean container (culture III).

Hindguts of three adult insects from culture I were removed, cut into pieces, and pooled in anaerobic buffer solution (13). During dissection, a continuous stream of nitrogen gas was applied to maintain anaerobic conditions. After confirmation of the presence of large numbers of *N. ovalis* organisms by inverted microscopy, the suspension was added to the autoclaved feed of insects in culture III. During the inoculation experiments, anaerobic buffer solution was added to the feed to produce a thick suspension.

(iv) Infection with a hindgut suspension devoid of protozoa (culture IV). Cockroach culture IV was derived from culture II to evaluate the effect on methane production and other parameters of feeding the insects a protozoan-free hindgut suspension. Hindguts of three adult cockroaches from culture I were pooled and cut into pieces in distilled water containing 2.1 mM $Na_2S \cdot 9H_2O$ and 1.8 mM cysteine-HCl (pH adjusted to 6.8 to 7.0). Microscopic observation revealed that *N. ovalis* cells burst because of osmotic shock and methanogenic endosymbionts were released. After we ascertained microscopically that no intact ciliate cells were present, the suspension was filtered through 7-μm-pore-size nylon gauze. The filtering was needed because preliminary experiments had demonstrated that cockroaches developed large numbers of *N. ovalis* organisms in their hindguts after addition of a nonfiltered, osmotic shock-treated suspension, most probably originating from cysts which had passed the treatment undamaged. The filtrate was mixed with the autoclaved substrates and fed to cockroaches in culture IV. At frequent intervals, the absence of *N. ovalis* was checked.

(v) Treatment with BES (cultures V and VI). To test the effect of specific inhibition of methanogens in *P. americana*, two cultures (V and VI) were started by transferring 20 adult insects from culture I into clean containers. The feed was the same as that given the stock culture, but BES, a specific inhibitor of methanogenesis, was added to the drinking water at concentrations of 25 and 50 mM in cultures V and VI, respectively. BES treatment was started only 3 weeks after transfer of the insects to their new containers. At frequent intervals, two or three insects from each culture were dissected for analyses of ciliate numbers and VFA concentrations in their hindgut contents. Methane production was analyzed by using five intact cockroaches at a time. Only adult insects were used for observations and collection of data.

BES treatment in vitro. To study the effects of different concentrations of BES on methanogenesis in in vitro cultures of *N. ovalis* and other hindgut microorganisms, three hindguts of adult cockroaches from culture I (65 μl per hindgut) were pooled and cut into pieces. A volume equivalent to about one-fourth of the original hindgut volume was transferred into 8-ml serum bottles which contained 3 ml of anaerobic buffer (pH 6.8 to 7.0). Ground cellulose (0.1%, wt/vol), ground grass (0.1%, wt/vol), and yeast extract (0.2%, wt/vol) were added to the buffer as organic substrates. BES was added to duplicate serum bottles to final concentrations of 0, 1, 2, 5, 10, and 20 mM, respectively. After the serum bottles were sealed and the headspace was flushed with nitrogen gas, the bottles were incubated at room temperature (25 to 28°C). Methane production was monitored daily, and the presence of methanogens and ciliates was checked for every other day. Twice a week, half of the culture medium of each bottle was replaced by fresh buffer and substrates. The culture media were removed through 7-μm-pore-size nylon gauze and used for VFA analysis. The in vitro cultures were maintained for 6-week periods.

Analysis of methane and VFA. Methane production by intact insects and by in vitro incubations of hindgut contents were analyzed gas chromatographically as described previously (13).

For VFA analyses, the pooled hindgut contents of two adult insects from each culture were used. One milliliter of buffer solution was always added, since the hindgut contents were usually semisolid. After mixing, samples were stored at −20°C until VFA analysis. VFA analysis of in vitro incubations was done on samples which were removed twice every week from serum bottles. Individual concentrations of acetate, propionate, and butyrate were analyzed by gas chromatography as described previously (14).

Microscopy. The presence of *N. ovalis* cells in hindgut samples or in in vitro incubations was checked by using a Leitz inverted light microscope. Ciliate enumeration was done as described earlier (13). The presence of methano-

TABLE 1. Numbers of ciliates, methane production, and body weights of insects in different cultures

Culture	Mean no. of *N. ovalis* organisms/ hindgut ± SD	Mean CH_4 production (μmol/ insect/day)[a] ± SD	Mean insect body wt (g) ± SD
I	3,500 ± 1,150	73.1 ± 7.8 (49.6)	1.47 ± 0.25
II	ND[b]	ND	0.68 ± 0.11
III	3,210 ± 228	64.8 ± 5.6 (71.1)	0.91 ± 0.13
IV	ND	17.2 ± 0.4 (23.6)	0.73 ± 0.13
V	3,605 ± 1,088	1.6 ± 0.1 (1.1)	1.51 ± 0.23
VI	3,850 ± 908	1.5 ± 0.1 (1.0)	1.49 ± 0.19

[a] Values in parentheses are micromoles of CH_4 per gram of body weight per day.
[b] ND, not detected.

genic endosymbionts and free-living methanogens was assessed by means of Leitz epifluorescence microscopy (10).

Statistical analysis. Statistical significance of differences between means was assessed by Student's *t* test.

RESULTS

The heterogeneous microbial population in the cockroach digestive tract is easily transferred from adults to newly hatched cockroaches by coprophagy (8) and possibly via feed or by direct contact. By rearing cockroaches strictly separated from the stock culture at the ootheca stage and feeding them with autoclaved substrates, adult cockroaches free of *N. ovalis* were obtained (culture II). Frequent microscopic examination of the hindgut contents of these insects revealed that they remained devoid of *N. ovalis* and other intestinal protozoa, e.g., flagellates, unless they were exposed to hindgut contents obtained from cockroaches in culture I via their feed (culture III). The absence of a normal intestinal microbial population in cockroaches had major effects on body weight and on methane production by adult insects (Table 1). No methane production was detected in cockroaches in culture II, and also no methanogens were observed in their hindguts by fluorescence microscopy. The average body weight of adult individuals was only about 50% of the weight of adult insects in culture I. Moreover, cockroaches in culture II developed hindguts with an average volume of only 43 ± 4 μl, which is substantially lower than the hindgut volume of normal insects, which was 65 ± 6 μl (significantly different at $P < 0.001$).

Microscopic observations showed that hindguts of insects from culture II were free of protozoa, but they were not germfree. Numerous bacteria were observed in these cockroaches. Flagellates were normally found to be present in the hindgut in high numbers (culture I) but were not observed in insects in culture II. To test whether the effects of prevention of the establishment of a normal protozoal population can be annihilated at adulthood, animals from culture II were treated with complete (culture III) and ciliate-free (culture IV) hindgut homogenates. Only hindgut microbial populations were considered in this study, because the hindgut has been shown to contain the most diverse and dense flora compared with other regions of the intestinal tract (2, 9). Moreover, it is likely that owing to the experimental setup contamination with foregut and midgut organisms was also effectuated in these trials.

After exposure of cockroaches from culture II to complete hindgut homogenates from insects in the stock culture, normal levels of methane production and ciliate numbers

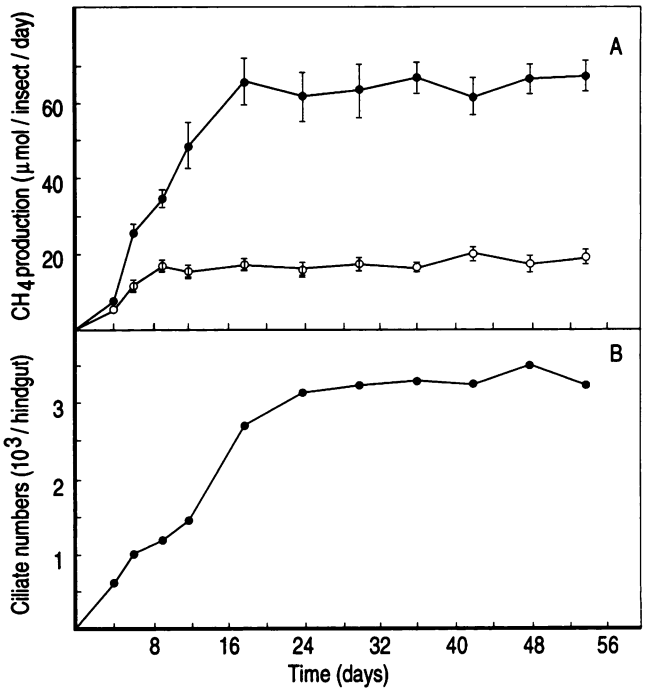

FIG. 1. Effect of feeding insects from culture II with a complete (●) or protozoan-free (○) hindgut suspension on methane production (A) and number of *N. ovalis* organisms (B).

were restored within only 2 weeks (Fig. 1). The average body weight of these insects increased to 0.91 g within 2 to 3 weeks, which was significantly different ($P < 0.001$) from that of cockroaches in culture II (Table 1). Even after 8 weeks, the weight of adult insects did not further increase to reach values equivalent to those of the stock culture. However, adult insects of the second generation in culture III attained an average body weight of 1.39 ± 0.24 g, which is similar to values obtained for culture I (Table 1). Although overall methane production by adult cockroaches in cultures I and III were almost identical, methane emission expressed per gram of insect fresh weight was higher in culture III, owing to the lower average body weight.

Earlier experiments in which *N. ovalis* was eliminated from insects by metronidazole demonstrated that small amounts of methane were still produced, most probably by free-living hindgut methanogens (13). Feeding of cockroaches derived from culture II with a hindgut homogenate made free of protozoa (culture IV) only restored methane production to about 20% of the production by adult insects in the stock culture (Fig. 1). Methane production in these cockroaches started to increase almost immediately after feeding with the hindgut suspension and reached stable values of 17.2 ± 0.4 μmol per insect per day within 10 days. The body weight of adult cockroaches in culture IV was not significantly different ($P > 0.005$) from that of those in culture II. Also, the second generation of cockroaches in culture IV showed poor development, with an average body weight at adulthood of only 0.8 ± 0.15 g. No *N. ovalis* cells or flagellates were observed at any time in the hindguts of cockroaches in culture IV.

Treatment of cockroaches originating from culture I with 25 or 50 mM BES in the drinking water resulted in almost immediate inhibition of methane production (Fig. 2). Meth-

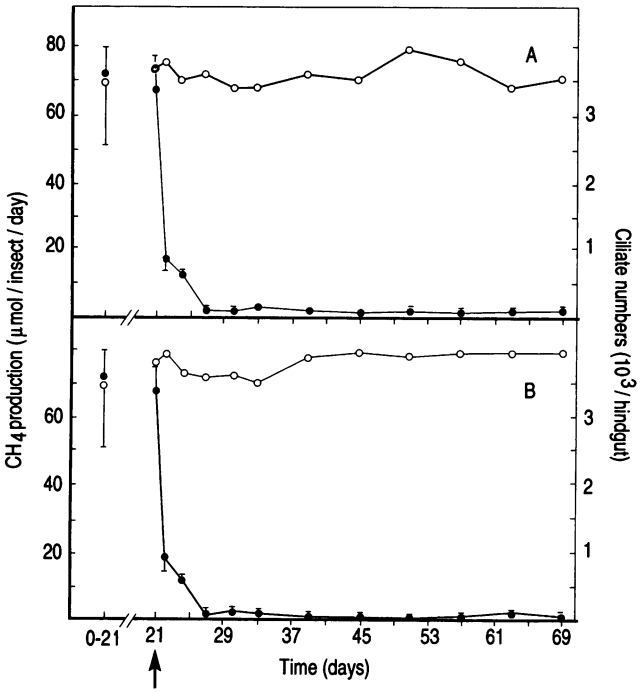

FIG. 2. Effect of 25 (A) or 50 (B) mM BES on methane production (●) and number of *N. ovalis* organisms (○). The arrow indicates the start of BES addition to the drinking water.

ane production in these cultures (V and VI) was lowered to about 2% of that of insects from the stock culture.

However, even after long-term exposure to BES (50 days), methanogens were not completely eliminated from the hindguts of cockroaches in cultures V and VI. When BES treatment was stopped, it took about 4 weeks before methane production was fully restored to values equivalent to those of culture I (Fig. 3).

BES treatment had no effect on insect body weight or number of *N. ovalis* cells in the hindgut (Table 1 and Fig. 2). Levels of acetate and butyrate in hindguts of BES-treated and control insects were almost identical, but there was a tendency toward higher propionate levels in BES-treated insects (Table 2).

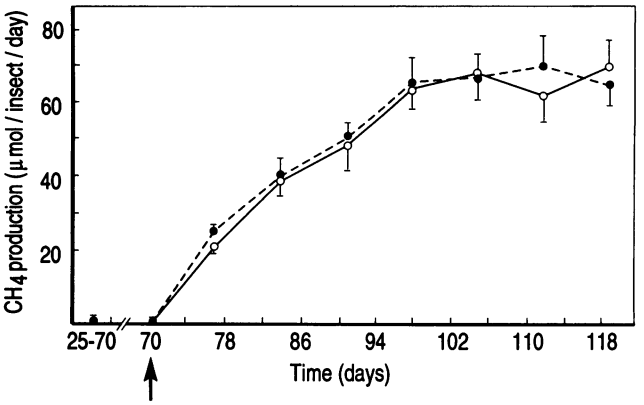

FIG. 3. Recovery of methane production after end of treatment with 25 (●) or 50 (○) mM BES. The arrow indicates the start of BES omission.

TABLE 2. Effect of BES on hindgut VFA concentration

Culture	BES concn (mM)	Mean VFA concn (mM) ± SD (n = 9)		
		Acetate	Propionate	Butyrate
I	0	18.7 ± 2.0	8.4 ± 0.6	4.7 ± 0.2
V	25	18.2 ± 1.7	10.0 ± 1.1	5.0 ± 0.3
VI	50	19.0 ± 1.0	10.3 ± 0.5	4.9 ± 0.3

Inhibition of methane production in in vitro incubations of hindgut suspensions was achieved at much lower BES concentrations than those used in intact insects. This was probably because of the more direct exposure to the drug in vitro. A concentration of 1 mM BES reduced methane production to about 1% of the production by control incubations without BES (Table 3). Complete inhibition of methanogenesis occurred at BES concentrations above 2 mM. BES treatment in vitro resulted in a substantial increase in propionic acid concentrations ($P < 0.001$), whereas production of acetate and butyrate remained almost constant at all of the BES concentrations applied.

N. ovalis was maintained in vitro over extended periods at stable numbers of 500 to 700 cells per ml (Table 3). Long-term exposure (6 weeks) to BES at concentrations of up to 20 mM did not affect ciliate numbers in these incubations. Preliminary experiments revealed that complete elimination of *N. ovalis* occurred at BES concentrations of 25 and 50 mM (data not shown), but this was probably a nonspecific effect.

As described earlier, fluorescence microscopy revealed the presence of numerous methanogenic bacteria as endosymbionts of *N. ovalis* (13). Also, in *N. ovalis* from culture I and III cockroaches, the typical bright blue-green fluorescence of methanogens (10) was visible. However, *N. ovalis* cells from cultures V and VI and from in vitro incubations which were treated with BES showed only faint fluorescence of endosymbionts. When BES treatment was stopped, normal fluorescence from both endosymbiotic and free-living methanogens was restored, indicating that methanogens remained viable.

DISCUSSION

In contrast to the situation in lower termites, in which hindgut bacteria and cellulolytic protozoa appear to be essential to insect survival (6, 11), cockroaches seem to be less strictly dependent on their hindgut microorganisms (9). However, our results demonstrate that cockroaches which developed from nymphs lacking a normal gut microbial population showed poor performance in terms of adult body weight, hindgut volume, and methane production. Moreover, the generation time of about 3.5 months observed for these insects was much longer than the average generation time of the parent culture (about 2.5 months; data not shown). The negative effects due to prevention of the establishment of a normal intestinal microbial population (culture II) were not completely restored by introducing hindgut microorganisms at adulthood. Earlier reports indicated that no adverse effects were observed when anaerobic intestinal microorganisms of *P. americana* were eliminated at adulthood by administration of a high dose of metronidazole (4). However, in these experiments drug-fed nymphs showed retarded development and smaller hindgut volumes, which corresponds to our findings on culture II.

The differences observed between cockroaches in cultures

TABLE 3. Effect of BES on in vitro cultures of hindgut microorganisms

BES concn (mM)	Mean VFA production (mmol/liter/day) ± SD (n = 10)			Mean CH_4 production (μmol/liter/day) ± SD	Mean no. of *N. ovalis* organisms/bottle ± SD
	Acetate	Propionate	Butyrate		
0	1.72 ± 0.16	0.82 ± 0.09	0.41 ± 0.05	5.70 ± 0.40	620 ± 150
1	1.73 ± 0.18	1.11 ± 0.05	0.42 ± 0.03	0.053 ± 0.013	530 ± 130
2	1.70 ± 0.20	1.23 ± 0.12	0.43 ± 0.05	0.013 ± 0.013	640 ± 160
5	1.72 ± 0.15	1.26 ± 0.07	0.41 ± 0.02	ND[a]	590 ± 112
10	1.73 ± 0.16	1.26 ± 0.09	0.41 ± 0.05	ND	655 ± 142
20	1.77 ± 0.13	1.23 ± 0.04	0.38 ± 0.08	ND	570 ± 105

[a] ND, not detected.

I and II might not be attributed solely to the absence of *N. ovalis* and methanogens, since other essential intestinal microorganisms may also have been absent in these insects. Flagellates, for instance, were normally observed in cockroach hindguts from culture I but were absent in insects in cultures II and IV. However, it is evident from our results, together with those of Bracke et al. (4), that the hindgut microbiota contributes significantly to the nutrition and development of *P. americana* during the early insect developmental stages. Comparison of second-generation cockroaches from cultures III (with protozoa) and IV (without protozoa) suggests that the retarded development can be ascribed mainly to the absence of hindgut protozoa.

Our experiments demonstrate that methane production also occurs in the absence of *N. ovalis* (culture IV), but at a much lower rate. The results confirm earlier findings that methanogenic endosymbionts of *N. ovalis* are the major source of methane production in *P. americana* (13). Kane and Breznak (15) recently reported that methane production in the hindgut of *P. americana* varied with diet and developmental stage. These investigators, however, could not detect ciliates in the gut contents of their cockroaches. It is interesting that methane production rates of about 5 μmol/g of body weight per day, as reported by Kane and Breznak (15), are even lower than the methane production of our cockroaches which were treated with a protozoan-free hindgut suspension (culture IV). Moreover, the average body weight of adults indicated by Kane and Breznak (15) appeared to be very similar to the low values observed in our cockroach cultures II and IV, which had no *N. ovalis*. This suggests that colonies of *P. americana* free of *N. ovalis* also occur in nature and that the relatively poor development and performance (in terms of adult body weight, hindgut volume, and methane production) of these insects may be due to the lack of hindgut ciliates. These findings support the idea that the presence of *N. ovalis* substantially contributes to the overall metabolism and nutrition of *P. americana* and therefore might be of both ecological and physiological importance to the host. It remains unclear why some colonies of *P. americana* apparently remain free of *N. ovalis* (15) whereas others develop large numbers in their hindguts (13; this study). Climatic influences, such as temperature, might be one parameter which affects the composition of the hindgut microbiota.

The presence of *N. ovalis* appears to be essential for high-rate methane production. However, ciliates showed no direct dependence on methanogens over the short period of the experiment. The results of this study show that inhibition of methanogens with BES did not affect the number of *N. ovalis* organisms in vivo or in vitro. Recently, Messer and Lee (17) reported the complete elimination of methanogens, associated with flagellates in termite hindguts, within 4 days after initiation of BES treatment. The hindgut protozoa and termite functioning did not appear to be affected by BES, which is in agreement with our findings on *P. americana*. Inhibition of methanogens, however, resulted in a relative increase in the level of propionate production. This shift in fermentation products was probably caused by increases in partial hydrogen pressure, resulting in the production of less-oxidized and less energy-yielding fermentation products, such as propionate.

Specific methane production levels (expressed per gram of body weight) in normal cockroaches (culture I) were high compared with those of other insects and even compared with those of ruminants, which are known to produce large amounts of methane. Methane production by the cockroach *Eublaberus posticus* was even higher than that of *P. americana* (8). Considering the relatively large amounts of methane produced by cockroaches (8, 13) and their large population size, especially in tropical areas, these insects might represent a potentially large source of atmospheric methane. Cockroaches, together with other methane-producing xylophagous insects, such as lower termites (12, 19) and scarab beetles (1), may account for a significant portion of global methane production.

The present study underscores the importance of nutritional interactions between *P. americana* and its anaerobic hindgut microbiota during the insect developmental stages. The results suggest a key role for *N. ovalis* in cockroach metabolism. Studies using axenic cultures of *N. ovalis* should be useful in providing further information on the biochemical activities of this protozoal symbiont.

ACKNOWLEDGMENTS

We thank G. D. Vogels and C. K. Stumm from the Department of Microbiology, University of Nijmegen, Nijmegen, The Netherlands, for valuable discussions and for critically reading the manuscript.

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